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(Alpha)6 (BETA)4

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13. ABSTRACT (Maximum 200 words) <p>Breast carcinoma invasion is a complex process in which the normal balance of cellular adhesion, proteolysis and directed migration is altered leading to penetration of the basement membrane and underlying stroma. An understanding of the mechanism of carcinoma cell invasion is critical to the diagnosis, control, and eventual treatment of late stage breast cancer. Work from our lab has shown that expression of the integrin <math>\alpha 6 \beta 4</math> in breast carcinoma cells enhances their invasiveness. This finding demonstrates that the integrin <math>\alpha 6 \beta 4</math> regulates signaling pathways important in tumor invasion and offers an excellent model for studying these pathways. My data show that integrin <math>\alpha 6 \beta 4</math> expression in breast carcinoma cells leads to an increase in chemotaxis toward fibroblast conditioned medium or lysophosphatidic acid (LPA). Additionally, this integrin <math>\alpha 6 \beta 4</math>-dependent chemotaxis is accompanied by the formation of lamellipodia. Both lamellae formation and chemotactic migration are inhibited or 'gated' by cAMP. My results reveal that a critical function of <math>\alpha 6 \beta 4</math> is to suppress the intracellular cAMP concentration by increasing the activity of a rolipram-sensitive, cAMP-specific phosphodiesterase (PDE). Also, my results show that RhoA controls lamellipodial formation and is required for directed migration. <b>I hypothesize that integrin <math>\alpha 6 \beta 4</math> amplifies signals required for lamellipodial formation thereby enhancing chemotaxis.</b> The goal of this study is to understand how integrin <math>\alpha 6 \beta 4</math> enhances lamellipodial formation and chemotaxis in breast carcinoma cells by identifying the signaling pathways involved in these processes.</p>				
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## Introduction:

Tumor cell invasion and subsequent metastasis pose a serious threat to the survival of breast cancer patients. We currently need a better understanding of invasion in order to manage and eventually treat the late stages of breast cancer. The process of invasion requires a delicate balance between cellular adhesion, proteolysis and directed migration. In this process, cells must degrade the basement membrane and migrate into the surrounding stroma (13). While basement membrane must be degraded proteolytically during invasion, recent studies have shown that many invasive tumors maintain a certain degree of integrity to the basement membrane (2, 10, 19, 20, 27) and that the basement membrane is an active rather than a passive participant in the process of invasion (5, 7, 27). Laminin, a major component of the basement membrane, is important for the adhesion and migration of carcinoma cells (26).

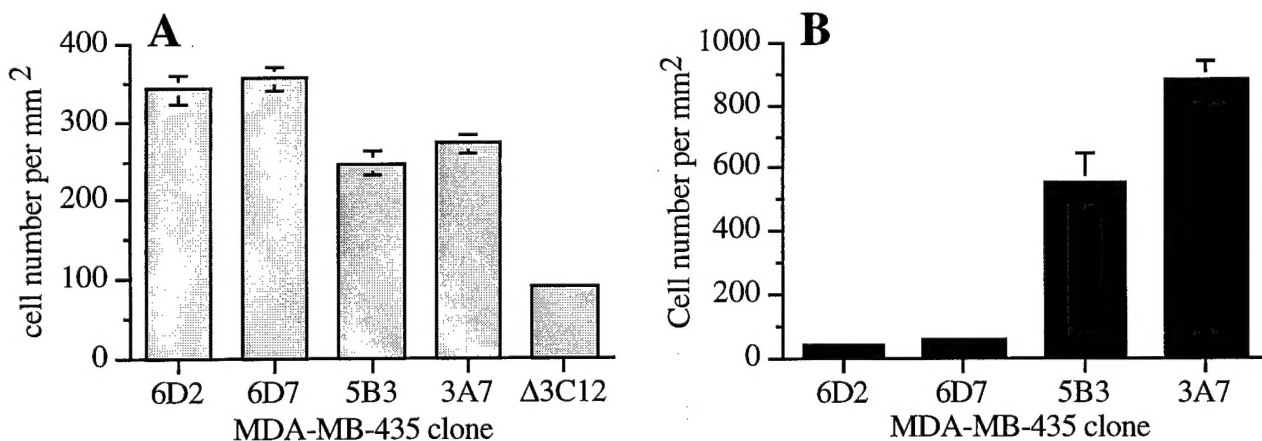
The integrin  $\alpha 6 \beta 4$ , a receptor for laminin-1 (12), laminin-2, laminin-4 (25), and laminin-5 (17), has been linked to acquisition of an invasive phenotype and progression in multiple cancers (6, 21, 28). With breast cancer, recent studies have formed a strong link between integrin  $\alpha 6 \beta 4$  and breast carcinoma invasion. First, Marchisio et al. have observed a striking localization of integrin  $\alpha 6 \beta 4$  in invasive breast carcinoma. Also, they detect the  $\beta 4$  integrin in the sera of women with metastatic breast cancer but not in the sera of healthy women (3). These observations support a previous study finding that expression of the integrin  $\alpha 6$  subunit is more strongly associated with reduced patient survival than other markers including the estrogen receptor (8). Therefore, I believe that the integrin  $\alpha 6 \beta 4$  plays a critical role in breast carcinoma invasion and progression and that the mechanism involved needs to be investigated.

Using an *in vitro* invasion assay system (1), our group has shown that integrin  $\alpha 6 \beta 4$ , can enhance the invasive potential of MDA-MB-435 breast carcinoma (24) and the RKO colon carcinoma (4) cell lines. Both the MDA-MB-435 and RKO cells express the laminin receptor integrin  $\alpha 6 \beta 1$ . Transfection of the integrin  $\beta 4$  subunit in these carcinoma cells results in the surface expression of the integrin  $\alpha 6 \beta 4$  and increases the invasiveness of these cells. Furthermore, the expression of the integrin  $\alpha 6 \beta 4$  does not lead to increased haptotactic migration on laminin for either the breast or colon carcinoma cell line. Since tumor cell invasion requires a delicate balance between cellular adhesion, proteolysis and directed migration, the integrin  $\alpha 6 \beta 4$  signaling in this process likely involves the stimulation of one of these processes. I have recently resolved this issue by discovering that expression of the integrin  $\alpha 6 \beta 4$  dramatically stimulates breast carcinoma cell chemotaxis, the directed migration toward a soluble attractant gradient, but not the proteolytic activity of these cells.

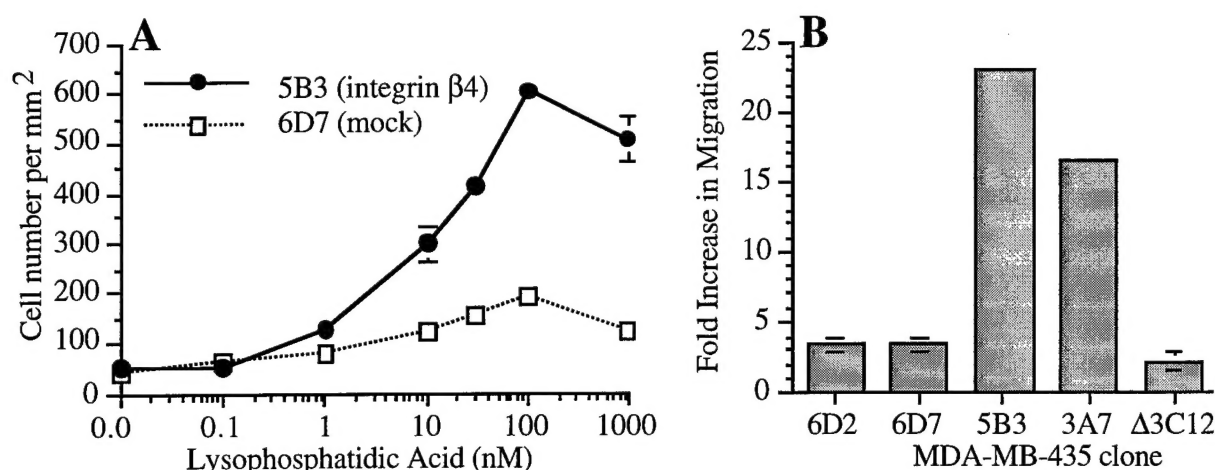
Currently, little is known about how integrin signaling impacts carcinoma cell chemotaxis and tumor progression. *Ultimately, the results from this study should help to delineate the role of  $\alpha 6 \beta 4$  integrin in facilitating chemotaxis and shed light on general signaling mechanisms that lead to a more invasive phenotype that characterizes late stage breast cancer.* Toward this goal, I have made considerable progress during this first year of this fellowship.

**Body:**

To study the effect of the integrin  $\alpha 6 \beta 4$  on breast carcinoma cell chemotaxis, I used MDA-MB-435 human breast carcinoma cells, which do not express the  $\alpha 6 \beta 4$  integrin (MDA/mock), as well as stable transfectants that express integrin  $\alpha 6 \beta 4$  (MDA/ $\beta 4$ ) or  $\alpha 6$  associated with the cytoplasmic domain deletion mutant of the  $\beta 4$  subunit (MDA/ $\beta 4$ - $\Delta$ CYT). Both the  $\beta 4$  transfectants and the  $\beta 4$ - $\Delta$ CYT transfectants expressed the  $\alpha 6 \beta 4$  heterodimer on the cell surface as assessed by FACS analysis and immunoprecipitation of surface-labeled extracts. As shown in Figure 1B, expression of integrin  $\alpha 6 \beta 4$  (clones 5B3 and 3A7) induces a 10-15 fold increase in the rate of chemotaxis toward NIH3T3 conditioned medium as compared to mock transfected MDA-MB-435 cells (clones 6D2 and 6D7). This expression of integrin  $\alpha 6 \beta 4$  does not alter laminin haptotaxis significantly (Figure 1A) suggesting that the stimulating effect of this integrin is specific for chemotaxis. To determine the factor in the conditioned medium that stimulates chemotaxis, I tested several factors that have been reported to stimulate chemotaxis including epidermal growth factor, basic fibroblast growth factor, hepatocyte growth factor/scatter factor, insulin-like growth factor I, transforming growth factor  $\alpha$  and  $\beta$ , platelet-derived growth factor (AA and BB), somatostatin, thrombin and lysophosphatidic acid (LPA). Of these factors, only LPA stimulated chemotaxis in an integrin  $\alpha 6 \beta 4$ -dependent manner. Maximal stimulation of chemotaxis was observed at an LPA concentration of 100nM (Figure 2A). LPA-stimulation of chemotaxis is 5 to 7 fold greater in the  $\beta 4$  transfectants than in the mock transfectants (Figure 2B). Subclones of the  $\beta 4$ - $\Delta$ CYT transfectants (e.g.  $\Delta$ 3C12) exhibit a rate of chemotaxis that is similar to the mock transfectants (Figure 2B) indicating the  $\beta 4$  cytoplasmic domain is critical for mediating the increased chemotaxis seen in the full-length  $\beta 4$ -transfectants. *My results show that expression of the  $\alpha 6 \beta 4$  integrin promotes chemotactic migration toward 3T3 conditioned medium and LPA without altering haptotactic migration.*

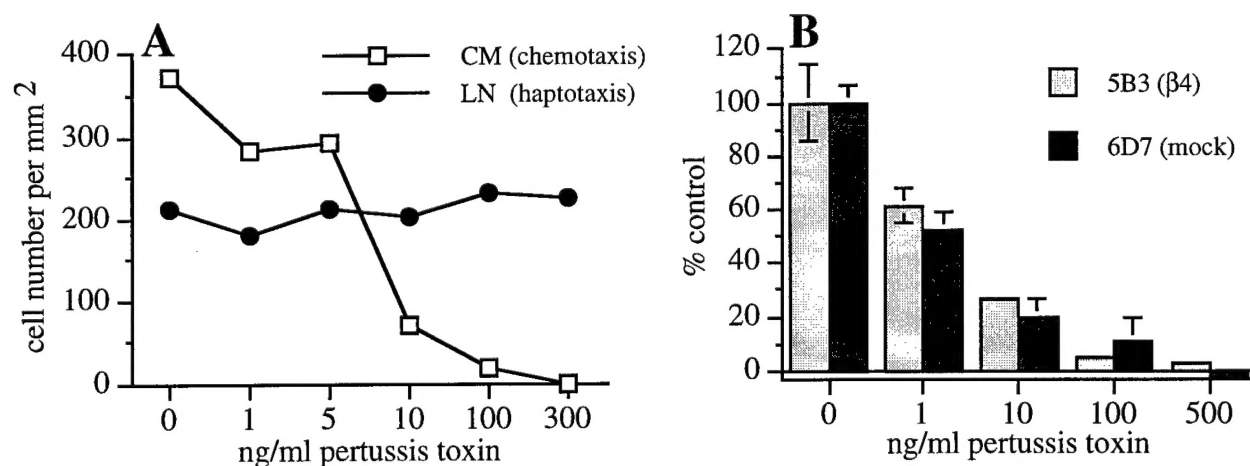


**Figure 1.** Integrin  $\alpha 6 \beta 4$  expression in MDA-MB-435 breast carcinoma cells stimulates chemotaxis but not haptotaxis. The migration of MDA-MB-435 cells which do not express  $\alpha 6 \beta 4$  integrin (clones 6D2, 6D7), express wild type  $\alpha 6 \beta 4$  integrin (clones 5B3, 3A7) or express  $\beta 4$  subunit cytoplasmic deletion mutant (clone  $\Delta$ 3C12) toward laminin (haptotaxis; A) or 3T3 conditioned medium (chemotaxis; B) was assessed using a modified Boyden chamber. The lower chamber of the Transwell dish was precoated with either laminin-1 or conditioned medium and then 100,000 cells were placed in the upper chamber. After 4 hours at 37°C, cells that did not migrate were removed from the upper chamber with a cotton swab and cells on the opposite side of the membrane were fixed, stained, and quantified manually as described previously (23). Data shown are representative of four experiments. Bars represent standard deviation from triplicate determinations.



**Figure 2.** Integrin  $\alpha 6 \beta 4$  amplifies LPA stimulated chemotaxis in MDA-MB-435 cells. (A)  $5 \times 10^4$  cells of MDA-MB-435 subclones 5B3 ( $\beta 4$  transfected; filled circles) or 6D7 (mock transfected; open squares) were placed in the upper chamber of collagen type I coated Transwell dishes and assayed as described in Figure 1 using various concentrations of LPA as a chemoattractant. (B) MDA-MB-435 cells transfected with either vector alone (6D2, 6D7), full length  $\beta 4$  integrin subunit (5B3, 3A7), or  $\beta 4$ - $\Delta$ CYT mutant ( $\Delta 3C12$ ) were assayed as described in (A) using 100nM LPA as a chemoattractant. Data are reported as fold increases over migration on collagen in the absence of LPA. Bars (A & B) represent standard deviation from triplicate determinations.

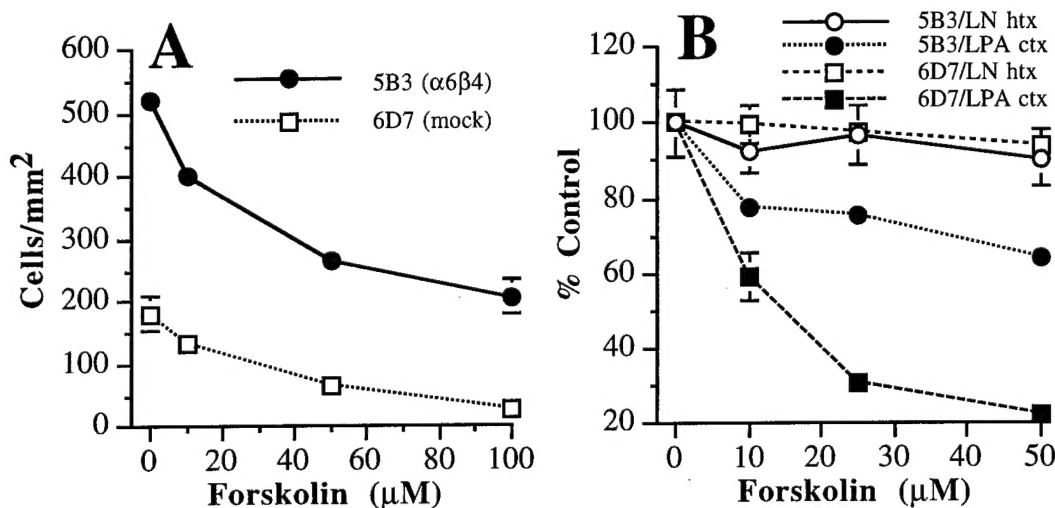
LPA is a bioactive phospholipid that can mediate its effects on cells through a heterotrimeric G-protein linked receptor that is linked to multiple G-proteins including inhibitory G ( $G_i$ ) proteins (16). To assess the possible involvement of a  $G_i$  protein in LPA-stimulated chemotaxis, I used pertussis toxin, which is known to inactivate heterotrimeric  $G_i$ -proteins by ADP ribosylation. As shown in Figure 3, conditioned medium- and LPA-stimulated chemotaxis are inhibited in a dose dependent manner by pertussis toxin. Moreover, the inhibition of chemotaxis observed at higher concentrations of toxin is not the result of cell toxicity because these concentrations of toxin did not inhibit haptotaxis (Figure 3A). *These data implicate a pertussis toxin sensitive  $G_i$ -protein in the LPA-stimulated chemotaxis of MDA-MB-435 cells.*



**Figure 3.** Chemotaxis, but not haptotaxis, is inhibited in a dose dependent manner by pertussis toxin. MDA-MB-435 cells transfected with full length  $\beta 4$  integrin subunit (3A7, 5B3) or vector alone (6D7) were treated with the indicated concentration of pertussis toxin for 30 min. prior to use in (A) CM chemotaxis (open squares), laminin haptotaxis (LN, closed circles) or (B) LPA chemotaxis assay as described in Figure 2B. The dose dependent decrease in chemotaxis was comparable in both the  $\beta 4$  and mock transfectants. This inhibition was not due to cytotoxicity since these concentrations of pertussis toxin did not inhibit haptotaxis toward laminin (A). Data in (B) are shown as the percent migration in the absence of pertussis toxin treatment minus background migration in the absence of LPA. Bars represent standard deviation of triplicate determinations.



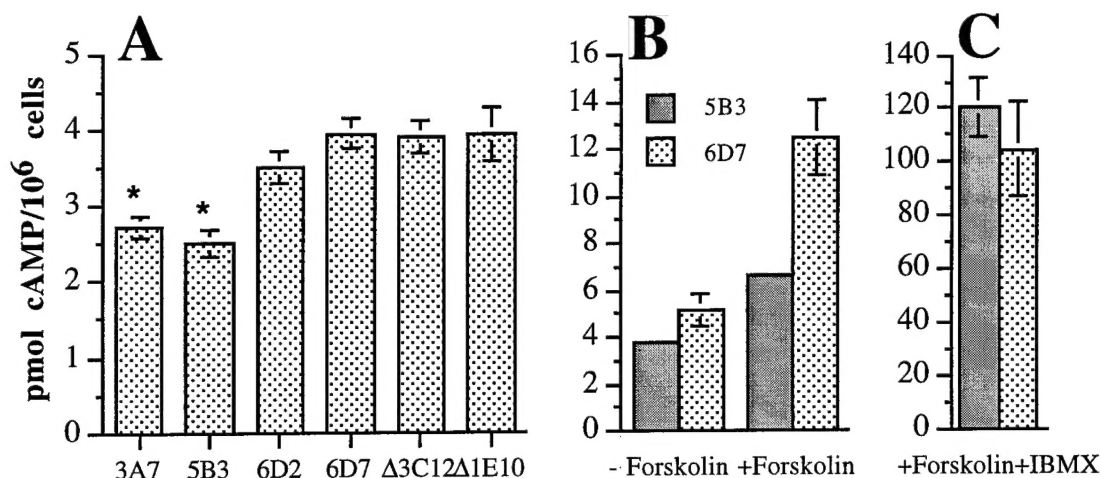
Because heterotrimeric G-proteins signal through adenylyl cyclase, I analyzed the role of adenylyl cyclase in chemotaxis using forskolin. As shown in Figure 4, forskolin stimulation of adenylyl cyclase inhibits LPA-stimulated chemotaxis. However, the  $\beta 4$ - and mock-transfected MDA-MB-435 cells differ in their response to forskolin. LPA-stimulated chemotaxis of the mock transfectants is inhibited completely by 50  $\mu\text{M}$  forskolin. At this concentration, the inhibition of chemotaxis of the  $\beta 4$ -transfectants is only 50% and higher concentrations of forskolin (100  $\mu\text{M}$ ) do not abrogate chemotaxis of these cells completely. Interestingly, treatment of the MDA/ $\beta 4$  or mock transfectants with forskolin did not inhibit haptotactic migration on laminin-1 (Figure 4B). *These data indicate that a cAMP-sensitive pathway plays a key role in LPA-stimulated chemotaxis of MDA-MB-435 cells and they suggest that the  $\alpha 6\beta 4$  integrin may regulate this pathway.*



**Figure 4.** Forskolin stimulation of adenylyl cyclase inhibits LPA mediated chemotaxis differentially in the MDA/ $\beta 4$  and mock transfectants. (A) MDA/ $\beta 4$  transfectants (5B3; solid circles) or mock transfectants (6D7; open squares) were treated with the indicated concentration of forskolin for 30 min. prior to their addition to the upper wells of the Transwell chambers. Cells were assayed for LPA-mediated chemotaxis on collagen I as described in Figure 1. (B) In a separate experiment, the same cells were treated with forskolin for 30 min. prior to assaying for LPA chemotaxis (filled symbols) or laminin haptotaxis (open symbols). Data are reported as the percent migration of cells not treated with forskolin  $\pm$  standard deviation of triplicate determinations.

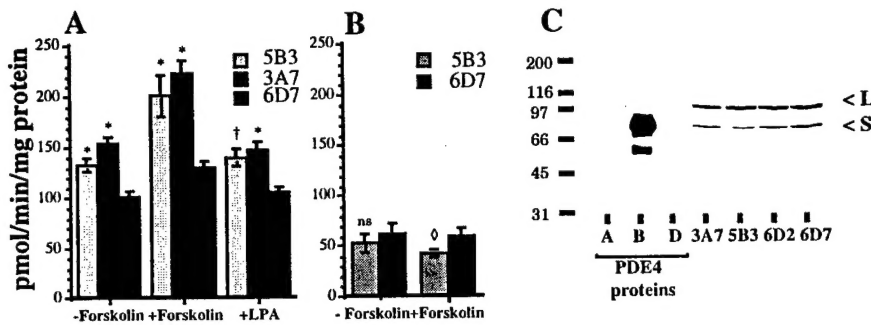
The forskolin data shown in Figure 4 suggest the concentration of cAMP may regulate the chemotactic response of MDA-MB-435 cells. The relative resistance of the  $\beta 4$ -transfectants to forskolin-inhibition of chemotaxis could result from  $\alpha 6\beta 4$ -mediated inhibition of adenylyl cyclase or  $\alpha 6\beta 4$ -stimulated degradation of cAMP. Both possibilities implicate a role for  $\alpha 6\beta 4$  in regulating cAMP levels. To determine if  $\alpha 6\beta 4$  expression influenced cAMP levels, I assayed the steady-state levels of cAMP in the mock and  $\beta 4$ -transfectants. As shown in Figure 5, the  $\beta 4$  transfectants had a lower steady-state level of cAMP than either the mock transfectants or  $\beta 4$ - $\Delta\text{CYT}$  transfectants and this difference was statistically significant ( $p < 0.001$ ). With forskolin stimulation, a 2.5-fold greater accumulation of cAMP was observed in the mock transfectants (6.6 pmol/ $10^6$  cells) compared to the  $\beta 4$  transfectants (2.6 pmol/ $10^6$  cells). When the forskolin-treated cells were also treated with the phosphodiesterase (PDE) inhibitor, IBMX, to prevent breakdown of cAMP, the MDA/ $\beta 4$  transfectants exhibited a  $[\text{cAMP}]_i$  comparable to the mock transfectants ( $120 \pm 11$  vs.  $104 \pm 18$  pmol/ $10^6$  cells, respectively; Figure 5C). *These data suggest that expression of  $\alpha 6\beta 4$  integrin suppresses the  $[\text{cAMP}]_i$  by increasing PDE activity.*





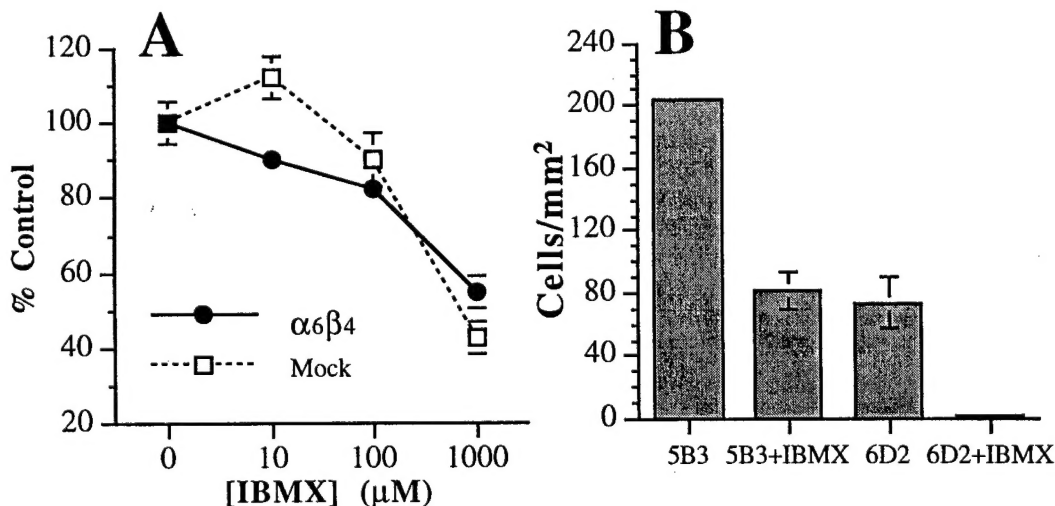
**Figure 5.** Intracellular cAMP content of the MDA-MB-435 transfectants. The MDA/ $\beta$ 4 (3A7, 5B3), MDA/ $\beta$ 4- $\Delta$ CYT ( $\Delta$ 3C12,  $\Delta$ 1E10) and MDA/mock transfectants (6D2, 6D7) were plated in DMEM containing 10% FCS. After 18 hrs., cells were harvested and cAMP content was measured using a cAMP EIA protocol (18). Data shown represent the mean of 10 sample determinations  $\pm$  standard error. The difference in the [cAMP]<sub>i</sub> between the MDA/ $\beta$ 4 and the mock transfectants is significant ( $p < 0.001$ ; denoted by asterisk), but the difference between the mock and the  $\beta$ 4- $\Delta$ CYT transfectants is not significant ( $p = 0.2$ ). (B, C) Differential effects of forskolin stimulation on the [cAMP]<sub>i</sub> in the MDA/ $\beta$ 4 and mock transfectants. The [cAMP]<sub>i</sub> was assayed in the 5B3 (solid bars) and 6D7 (stippled bars) clones plated on collagen I and treated for 15 min. with either 50  $\mu$ M forskolin (B) or forskolin and 1 mM IBMX (C). Note that the MDA/ $\beta$ 4 transfectants (5B3) are more resistant to a forskolin-stimulated increase in [cAMP]<sub>i</sub> than the mock transfectants (6D7). The inhibition of PDE activity with IBMX shown in C reveals that  $\alpha$ 6 $\beta$ 4 expression results in an increase in PDE activity and not a decrease in cAMP synthesis. Data shown are the mean values  $\pm$  standard error obtained from multiple experiments.

To establish more directly that expression of the  $\alpha$ 6 $\beta$ 4 integrin can regulate cAMP-dependent PDE activity, the activity of this enzyme was assayed in cell extracts obtained from the MDA/mock and  $\beta$ 4 transfectants. As shown in Figure 6A, the MDA/ $\beta$ 4 transfectants exhibited a significantly higher rate of PDE activity than the mock transfected cells. Moreover, the PDE activity of the MDA/ $\beta$ 4 transfectants was markedly increased (51% for 5B3 and 45% for 3A7) in response to forskolin stimulation compared to the mock transfectants (29% for 6D7; Figure 6A). The difference in PDE activity between the MDA/ $\beta$ 4 and mock transfectants was eliminated by rolipram, a type IV PDE-specific (PDE 4) inhibitor (Figure 6B). To examine the possibility that the MDA/ $\beta$ 4 and mock transfectants differed in their level of PDE expression, I assessed PDE 4 expression in these cells using antibodies specific for the various PDE 4 variants (9). The predominant PDE 4 variant expressed in MDA-MB-435 cells is PDE 4B based on results obtained with antibodies specific for PDE 4A, 4B and 4D (data not shown). Importantly, the expression of PDE 4B did not differ significantly between the MDA/ $\beta$ 4 and mock transfectants (Figure 6C). *These data indicate that the increased PDE activity observed in the MDA/ $\beta$ 4 transfectants is not the result of increased PDE expression.*



**Figure 6.** Assay of cAMP-specific PDE activity. (A) MDA/β4 (3A7, 5B3) or mock transfectants (6D7) plated on collagen I were treated with 50 μM forskolin or 100nM LPA as noted. Cells were harvested and the cytosolic fraction was assayed for PDE activity as described (18). The PDE activity of the MDA/β4 transfectants was compared to the MDA/mock transfectants for statistical significance: (\*) p-value < 0.002; (†) p-value < 0.01 (B) Extracts from cells treated as in A were incubated with 100μM rolipram prior to assaying for PDE activity to determine how much of the activity in (A) constitutes cAMP-specific PDE (PDE 4). Data shown are mean ± standard error of 4 separate determinations (A, B). (ns) not significant; (◇) p-value = 0.02 (C) Relative expression of PDE 4B in the MDA-MB-435 transfectants. Extracts (40μg protein) obtained from the MDA/β4 (3A7, 5B3) and mock (6D2, 6D7) transfectants, as well as purified PDE 4 proteins (short form of variants A, B, and D; 10ng each; provided by Marco Conti) were resolved by SDS-PAGE and immunoblotted with a PDE 4B specific Ab. Arrows to the right denote the long and short forms of PDE 4B.

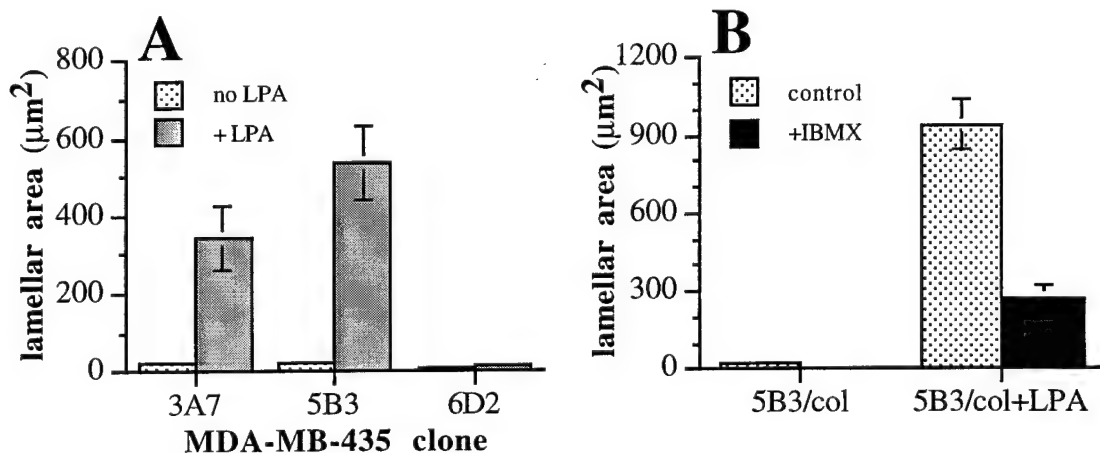
**PDE activity is necessary for chemotaxis, invasion, and lamellae formation:** The importance of PDE for chemotactic migration was examined by treating the MDA/mock and β4 transfectants with IBMX prior to their use in the chemotaxis assay. As shown in Figure 7A, IBMX inhibited LPA-stimulated chemotaxis with maximal inhibition observed at 1mM. Similar results were obtained with the cAMP-specific PDE inhibitor, rolipram (data not shown). I also examined the involvement of PDE in carcinoma invasion by treating cells with IBMX prior to their use in a standard Matrigel invasion assay. A substantial inhibition of invasion was observed in the presence of IBMX in comparison to the solvent control (Figure 7B). *These data show that the cAMP-specific PDE, which is stimulated by the α6β4 integrin, is critical for LPA-stimulated chemotaxis and invasion.*



**Figure 7.** cAMP specific-PDE activity is required for the chemotactic migration and invasion of MDA-MB-435 cells. The MDA/β4 (5B3; squares) or mock transfectants (6D7; circles) were treated with varying concentrations (A) or 1mM (B) IBMX for 30 min. prior to their use in an LPA chemotaxis assay (A) or a Matrigel chemoinvasion assay (B). Data shown represent mean values ± standard deviation of triplicate determinations.

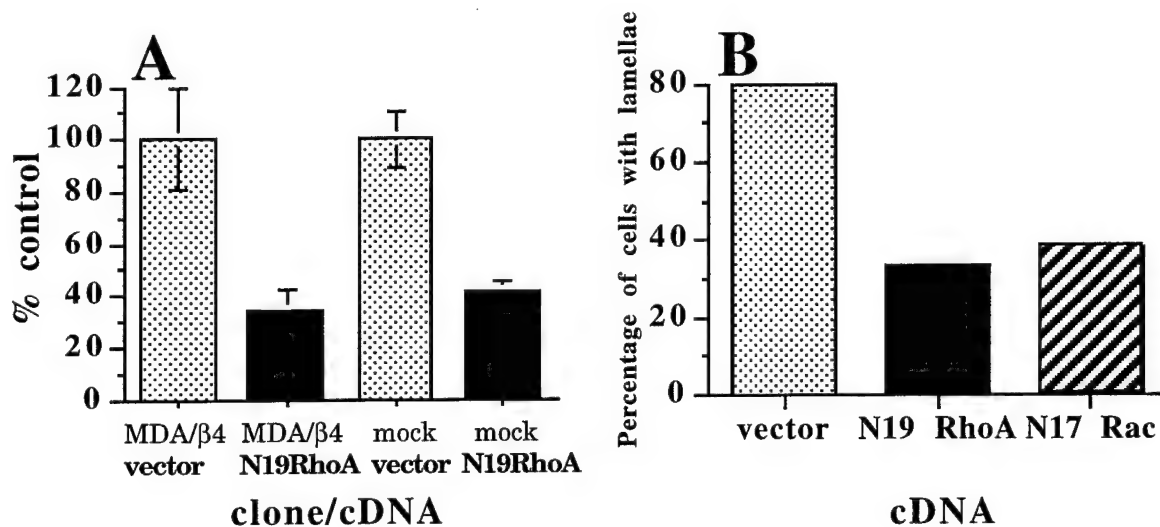
**Expression of the  $\alpha 6 \beta 4$  integrin is required for the formation of lamellae in response to LPA:** Chemotactic migration frequently involves the formation of broad sheets of polymerized actin at the leading edge of the cell termed lamellae (15). To determine if expression of the  $\alpha 6 \beta 4$  integrin influenced the formation of such motile structures, I analyzed the morphology of the MDA-MB-435 transfectants plated on collagen I. Prominent lamellae were not evident in the mock transfectants and treatment with 100 nM LPA did not stimulate a significant increase in lamellar area (Figure 8A). The MDA/ $\beta 4$  transfectants exhibited a similar morphology to that of the mock transfectants when plated on collagen I or laminin-1 (data not shown). Within minutes after LPA treatment, however, the MDA/ $\beta 4$  transfectants formed large, ruffling lamellae (data not shown). Quantification of these cells by digital image analysis indicated that LPA stimulated a dramatic increase in the lamellar area of the two subclones of the MDA/ $\beta 4$  transfectants (Figure 8A). In contrast, no increase in the lamellar area of the mock transfectants in response to LPA was detected by this analysis (Figure 8A).

The necessity of cAMP-specific PDE activity in the formation of lamellae was also assessed. IBMX had no effect on the morphology of the MDA/ $\beta 4$  transfectants in the absence of LPA (data not shown). However, IBMX-treated cells were unable to form the large, ruffling lamellae in response to LPA stimulation in comparison to untreated cells (data not shown). Quantitative analysis of these cell populations revealed that inhibition of PDE activity resulted in an approximate four-fold reduction in the lamellar area of LPA-stimulated MDA/ $\beta 4$  transfectants (Figure 8B). *Therefore, my result show that expression of the  $\alpha 6 \beta 4$  integrin promotes lamellae formation and that this lamellae formation requires cAMP-specific PDE activity.*



**Figure 8.** The  $\alpha 6 \beta 4$  integrin expression promotes LPA-dependent formation of lamellae in MDA-MB-435 cells and requires cAMP specific-PDE activity. (A) MDA/ $\beta 4$  (clones 3A7 and 5B3) and MDA/mock (6D2) were plated onto coverslips that had been coated with 20μg/ml collagen I. Cells were allowed to adhere for 2hrs at 37°C and then treated with LPA for 5 min. or left untreated. Lamellae were visualized using both phase contrast optics and FITC-phalloidin staining. The effect of LPA on lamellar area was quantified using IPLab Spectrum imaging software. using the criteria for defining lamellae used previously by our group (22). Lamellae were defined as broad, flat cellular protrusions rich in F-actin and devoid of membrane-bound vesicles. (B) The MDA/ $\beta 4$  transfectants (5B3) were plated on collagen I-coated coverslips. After 2 hours, the cells were either left untreated or treated with 1mM IBMX for 30 min. Subsequently, the cells were either left untreated or treated with 100nM LPA for 5 min. The effect of LPA and IBMX on lamellar area was quantified as in (A). Bars (A, B) represent mean lamellar area  $\pm$  standard error in which  $n > 20$ . Of note, IBMX inhibited the LPA-dependent formation of lamellae by 70%.

Of particular relevance to my work, Butcher and colleagues reported that cAMP is a negative regulator of leukocyte migration signaled through the classical chemoattractants (11). In this model, cAMP impedes or gates RhoA-mediated leukocyte integrin activation and adhesion. Since LPA is a potent activator of RhoA (16), I wanted to explore the possibility that the integrin  $\alpha 6 \beta 4$  releases cAMP gating of LPA-mediated RhoA activation that may lead to increased chemotaxis and lamellae formation. To address this question, I transiently transfected the MDA-MB-435 clones with a dominant negative construct of RhoA (N19 mutant of RhoA) or an empty vector. These transfectants were then assayed for their ability to chemotax or form lamellae in response to LPA. As shown in Figure 9, expression of dominant negative RhoA in both the MDA/ $\beta 4$  and MDA/mock cells inhibited chemotactic migration compared to a vector only control (Figure 9A). Interestingly, this construct was also able to inhibit LPA stimulated lamellae formation in the MDA/ $\beta 4$  cells (Figure 9B). *My data suggest that RhoA is essential for chemotactic migration and lamellae formation.*



**Figure 9.** Dominant-negative RhoA inhibits chemotactic migration and lamellae formation in MDA-MB-435 cells. A) N19RhoA cDNA or vector alone were co-transfected with  $\beta$ -gal cDNA into MDA/ $\beta 4$  or MDA/mock cells. After 24 hours, cells were assayed for LPA-stimulated chemotaxis as described in Figure 1 and then stained for  $\beta$ -gal. Data are reported as number of  $\beta$ -gal staining cells migrated versus negative control (vector alone)  $\pm$  standard deviation of triplicate determinations. B) MDA/ $\beta 4$  cells were co-transfected with either vector, N19RhoA or N17Rac cDNAs and a GFP reporter construct. After 48 hours, cells were plated onto LN-1 coated coverslips, treated with 100nM LPA for 5 minutes and then fixed. The percentage of GFP-positive cells that had lamellae were enumerated and are reported as the percentage of the total cell number from a representative experiment. Of note, N19RhoA and N17Rac expressing cells that formed lamellae had much smaller lamellae than control cells.

To distinguish responses that are altered by integrin  $\alpha 6 \beta 4$  signaling, I investigated the potential of MDA-MB-435 transfectants to mobilize  $\text{Ca}^{++}$  in response to 10 $\mu\text{M}$  cyclopiazonic acid (CPA) determined by microspectrofluorometry on single cells loaded with the  $\text{Ca}^{++}$  sensitive fluorescent dye, fura-2, following the method of Marks and Maxfield (14). As shown in Figure 10, the MDA/ $\beta 4$  cells had a lower intracellular  $\text{Ca}^{++}$  concentration ( $[\text{Ca}^{++}]_i$ ) than either the MDA/mock or MDA/ $\beta 4 \Delta \text{CYT}$  clones. This increase in  $[\text{Ca}^{++}]_i$  was sensitive to the concentration of extracellular  $\text{Ca}^{++}$  suggesting that this response is due to a capacitive  $\text{Ca}^{++}$  influx current ( $I_{\text{CRAC}}$ ; data not shown). *This data suggests that expression of the  $\alpha 6 \beta 4$  integrin can alter mobilization of  $\text{Ca}^{++}$  by modulating  $I_{\text{CRAC}}$  channels.*

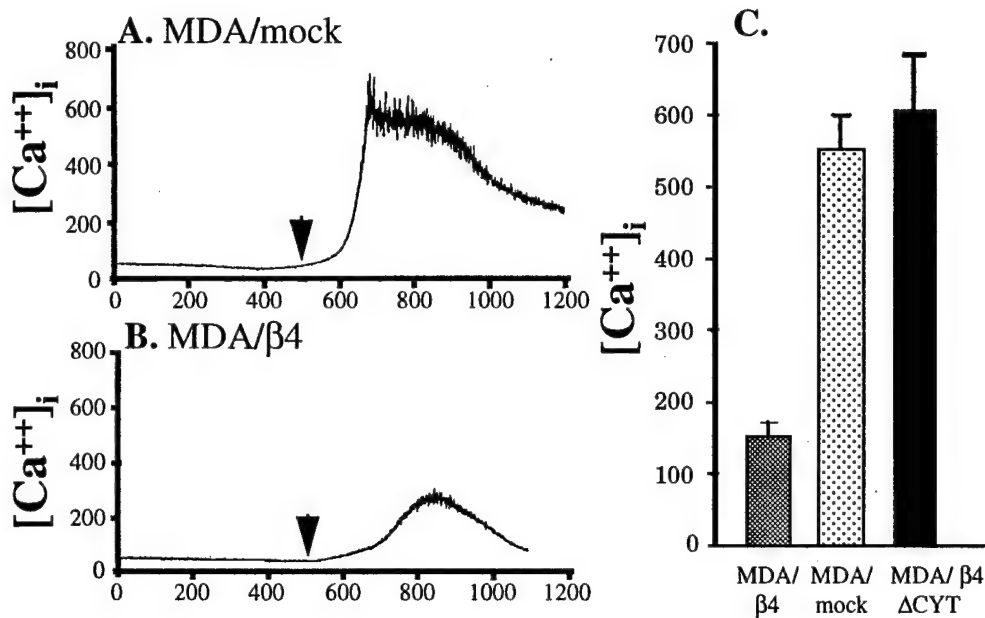


Figure 10. Expression of the  $\alpha 6 \beta 4$  integrin alters calcium mobilization in response to CPA. MDA/β4 (5B3) and MDA/mock (6D7) were plated onto coverslips that had been coated with 20 $\mu$ g/ml collagen I. Cells were allowed to adhere for 2hrs at 37°C and then loaded with 1mM FURA-2 for 30 min. Cells were then monitored at 380 and 340nm wavelengths. 380/340nm emissions ratios were converted to  $[Ca^{++}]$  using a standard curve. Representative  $[Ca^{++}]_i$  curves are shown for MDA/mock (A) and MDA/β4 transfectants (B). (C) Peak  $[Ca^{++}]_i$  values were averaged from multiple response curves. Bars represent mean  $[Ca^{++}]_i \pm$  standard error.

### Statement of works:

The  $Ca^{++}$  mobilization experiments were performed in collaboration with Dr. Hamid Akbarali, an electrophysiologist, who, until recently, was employed here in the Gastroenterology Division at Beth Israel Deaconess Medical Center. His recent transfer to the VA Medical Center has slowed these  $Ca^{++}$  mobilization studies (Task 1). Therefore, these studies will still be done but will take longer than originally expected. Arrangements have been made to continue these studies over the next year with Dr. Akbarali at the VA Medical Center.

The delay in the  $Ca^{++}$  studies has given me the opportunity to begin examining the activation of Rho family of GTPases by the integrin  $\alpha 6 \beta 4$  and assess their roles in lamellipodial formation and chemotaxis (Task 3) ahead of schedule.

### Conclusions:

During this first year of my fellowship, considerable progress has been made toward the understanding of the role of the  $\alpha 6 \beta 4$  integrin in breast carcinoma invasion. The data that I have obtained demonstrate that the  $\alpha 6 \beta 4$  integrin can stimulate lamellae formation and chemotactic migration of invasive carcinoma cells by increasing the activity of a rolipram-sensitive cAMP specific-PDE and lowering the  $[cAMP]_i$ . This cAMP specific-PDE functions in tandem with a PI3-K/Rac pathway, that is also regulated by  $\alpha 6 \beta 4$  (24), and is required for carcinoma invasion and lamellae formation. This work resulted in a publication in the Journal of Cell Biology (ref. 18).

I have also obtained evidence that the target of cAMP-gating is RhoA, as has been observed previously in leukocytes. I have preliminary data that suggests that RhoA is critical for chemotaxis and lamellipodial formation as determined through the use of a dominant negative RhoA construct. I have also initiated experiments to assess the effects of the integrin  $\alpha 6 \beta 4$  on calcium mobilization. I expect substantial progress will be made during the next year of my fellowship in these two areas of my study with the availability of new methods to analyze RhoA activity and the collaborative efforts of Dr. Hamid Akbarali on the calcium mobilization studies.

In summary, the data that I have obtained supports my hypothesis that the integrin  $\alpha 6 \beta 4$  amplifies signals required for lamellipodial formation that helps to promote chemotaxis. This fellowship has permitted me to investigate key signaling events that underlie carcinoma invasion and, I hope, will advance our understand and treatment of breast cancer.



**Final Reports:** The following are a list of publications and meeting abstracts that have resulted from the research efforts supported by this fellowship:

1. O'Connor, K. L., L.M. Shaw, and A. M. Mercurio. 1998. Release of cAMP gating by the  $\alpha 6 \beta 4$  integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *J. Cell Biol.* 143: 1749-1760.
2. O'Connor, K. L., L.M. Shaw , and A. M. Mercurio. 1998. Release of cAMP gating by the  $\alpha 6 \beta 4$  integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *Proc. Annu. Meet Amer. Soc. Cell Biol.* (meeting abstract).
3. O'Connor, K. L. and A. M. Mercurio. 1998. Integrin  $\alpha 6 \beta 4$  Promotes Lamellae Formation and the Chemotaxis of Invasive Carcinoma Cells by Releasing cAMP Gating of RhoA Activation. *The Function of Small GTPases: Keystone Symposium* (meeting abstract)

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